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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: August 9, 1973

1. Principal Investigator (give title and degrees):

R. G. Mason, M.D., Ph.D., Professor

2. Institution & address:

University of North Carolina
Chapel Hill, N. C. 27514

3. Department(s) where research will be done or collaboration provided:

Department of Pathology

4. Short title of study:

Effects of nicotine on interactions of platelets and endothelial cells

5. Proposed starting date: January 1, 1974

6. Estimated time to complete: December 31, 1976

7. Brief description of specific research aims:

We propose to test the hypothesis that nicotine influences the reactivity of endothelial cells with platelets and of platelets with each other. Research in four interrelated areas will be performed:

a. Comparison of ultrastructure, protein and glycoprotein components, and reactivity with platelets of endothelial cells recovered from human artery, peripheral vein or human umbilical cord and of those harvested from tissue culture.

b. Development and standardization of an in vitro model for endothelial cell-platelet adhesion. This model will be used in a study of the effects of nicotine, platelet aggregating agents, certain enzymes, and other agents on reactivity of endothelial cells with platelets. The basic approach will be to determine what is required to render endothelial cells attractive to platelets.

c. Investigation of possible "endothelial supporting" function of platelets and how this may be influenced by nicotine.

7. (cont.)

d. Characterization and further studies of an agent of endothelial cell origin which inhibits aggregation of platelets induced by a wide spectrum of agents. Effects of nicotine on the levels, release, and reactivity of this inhibitor will be studied.

e. In vivo studies of endothelial cell-platelet interaction in the microvasculature of hamster and spider monkey with investigation of effects of nicotine, aggregating agents, anticoagulants, antithrombotic agents, and certain enzyme preparations.

In all of the proposed studies, emphasis will be placed on correlation of function with ultrastructure. The investigations will be carried out by a group composed of workers with special training in the areas of cell biology, pathology, biochemistry, biophysics, and electron microscopy.

8. Brief statement of working hypothesis:

An understanding of the functions of endothelium in prevention of thrombosis should permit more effective control of this major health problem. Endothelium is thought to present a surface highly compatible with blood. Whether this property of endothelium resides in the molecular constitution of the outer layers of the cell's plasma membrane or is brought about by various inhibitors released by these cells, or both, is unknown. Knowledge of the molecular composition of the endothelial cell plasma membrane gained by disc electrophoretic studies of surface glycopeptides released by treatment with proteolytic enzymes should assist in synthesis of biomaterials more compatible with blood than those currently available. Possible effects of nicotine on membrane components of endothelial cells or platelets could alter their mutual reactivity. Similar benefit should derive from sodium dodecyl sulfate (SDS) gel electrophoretic studies of endothelial cell constituents which will characterize cell proteins and permit identification of those proteins exposed to the outer surfaces of the plasma membrane. Knowledge of conditions under which platelets may adhere to endothelial cells should permit identification of agents to inhibit the phenomenon. Comparison of studies with endothelial cells recovered from umbilical cord vein with endothelial cells grown in tissue culture should be most productive and helpful in the proposed studies since the former do not adhere to platelets while the latter are said to do so.[1] Finally, knowledge of substances of endothelial cell origin which inhibit platelet aggregation or adhesion and effects of nicotine upon these should prove

9. Details of experimental design and procedures (append extra pages as necessary)

A. Experimental design and procedures are listed in each of five interrelated areas of research.

1. Comparison of endothelial cells recovered from human artery, peripheral vein, and umbilical cord vein with endothelial cells grown in tissue culture. Endothelial cells from these 3 sources will be compared ultrastructurally and for differences in electrophoretic mobility, protein and glycoprotein composition, and reactivity with platelets in both the presence and absence of nicotine. These initial studies will be performed to delineate detectable differences between the three types of endothelial cells and detectable effects of nicotine. Only limited studies can be performed with cells recovered from artery or peripheral veins due to obvious difficulties in obtaining specimens within 1 to 2 hours after death. Human umbilical cords can be obtained easily in quantities of 2 to 3 per day, hence endothelial cells recovered from umbilical cord veins will be available for most studies. Endothelial cells of umbilical cord vein and of arterial or peripheral vein origin which have been grown in tissue culture will be available in numbers adequate for all proposed studies.

   Representative samples of the 3 types of endothelial cells will be examined in the presence and absence of nicotine by transmission electron microscopy (TEM) for comparison of ultrastructure. Comparison will also be made with the ultrastructure of endothelial cells in situ in various anteries, peripheral veins, and umbilical cord vein. The 3 types of endothelial cells will be compared also by freeze-etch (FE) techniques. Special attention in FE studies will be paid to the outer surface of the plasma membrane of each cell type and a careful search will be made for surface membrane associated particles.

   Use of endothelial cells recovered from umbilical cord vein by collagenase treatment will provide useful contrast with use of endothelial cells grown in tissue culture. Platelets do not adhere to endothelial cells recovered

8. (cont.)

valuable not only in furthering our understanding of mechanisms of thrombosis but also in our search for more effective antithrombotic agents for clinical use.

9. (cont.)

from umbilical veins even if aggregating agents are added, while they are reported to react with tissue culture endothelial cells under such conditions.[1] Effects of nicotine on cellular interactions will be determined. Comparison of protein and glycoprotein SDS gel disc electrophoretic patterns obtained from homogenates of each of the various types of endothelial cells will be carried out and effects of nicotine determined. This will permit detection of loss or gain of components of cells grown in tissue culture as well as detection of differences in patterns of samples from cells isolated by use of collagenase (umbilical cord vein cells) or by use of trypsin (vena cava cells). Further, proteins exposed on the outer surface of endothelial cells now can be labelled and identified.

The aggregometer will be used to compare further the reactivity of the 3 types of endothelial cells with platelets and effects of nicotine on their reactivity. Each endothelial cell type will be stirred at 1000 RPM at 37°C with citrated or heparinized platelet-rich plasma (PRP) or with plasma-free platelets suspended in Ca^{++}-Mg^{++}-free Tyrode's solution (modified Tyrode's solution or MTS). Mixtures will be checked photometrically for adhesion of endothelial cells to platelets during stirring and by phase-contrast microscopy at the termination of stirring.

Comparison of the 3 types of endothelial cells in the presence and absence of nicotine will provide information on at least some of the alterations which may occur in these cells as they are isolated from blood vessels and as they grow in tissue culture. This should permit more judicious use of tissue culture endothelial cells in future work.

Extension of studies of tissue culture endothelial cells and effects of nicotine in future years would include work to develop conditions for tissue culture growth of endothelial cells from adult venous and arterial segments from lung, liver, and spleen.

2. In Vitro Interactions of Endothelial Cells and Platelets.

a. Design and standardization of a model for endothelial cell-platelet interaction. The model consists of mixing endothelial cells in MTS with platelets in plasma or with isolated platelets in MTS. The mixture is stirred at 1000 RPM at 37°C in an aggregometer and changes in light transmitted through the mixture are detected by a photocell in the instrument. The following will be investigated:

1) Effects of alteration of speed of stirring over the range 100 to 2000 RPM.

2) Effects of alteration in temperature. Experiments will be conducted with cell mixtures at 15°, 23°, and 37°.

3) Effects of alteration in pH by use of appropriate buffers over the range pH 5-10 (barbital-sodium acetate buffer, pH 3-9; citrate-phosphate-borate buffer, pH 2-12; phosphate buffer, pH 5-8; imidazole buffer, pH 7.2-9).

4) Effects of alteration in ionic strength. Molar NaCl will be added in amounts sufficient to alter ionic strength over the range of normal to 3 times normal. One hundredth molar NaCl will be added in quantities sufficient to lower ionic strength over the range from normal to one-fourth normal.

5) Effects of various surfaces. Tests will be conducted in glass, glow discharge-treated glass, polyethylene oxide-treated glass, silicone-coated glass, polycarbonate, and styrene tubes and with Teflon, polyethylene, or poly (vinyl chloride)-covered stirring bars. Platelets are known to adhere to various artificial surfaces and such adhesion may produce release of various platelet components such as ADP and serotonin.

In each of the above, studies will be conducted both in the presence and absence of low levels of nicotine.

These studies should permit standardization of the model (hereafter referred to as the model system) and give us knowledge of which variables we should monitor most closely in future work.

b. Effects of platelet aggregating agents on endothelial cells and on interaction of platelets and endothelium. Endothelial cells of each type (cells isolated from umbilical cord vein or grown in tissue culture) will be incubated at 37°C without stirring with the following aggregating agents: ADP (10^{-3} to 10^{-7} M), collagen, epinephrine (10^{-3} to 10^{-7} M), serotonin (10^{-3} to 10^{-7} M), or thrombin (0.05 to 5 Iowa units/ml). Following this incubation period, the cells will be added directly to stirring PRP, except in work with higher levels or thrombin, or they will be washed 3 times in MTS and then added to stirring PRP to determine if adhesion of platelets to treated endothelial cells occurs. Likewise, platelets will be incubated with aggregating agents at 37°C without stirring and experiments similar to those described above will be performed. Platelet aggregation and platelet-endothelial cell adhesion will be detected by the aggregometer. Adhesion of platelets to endothelium will be evaluated also by phase-contrast microscopy. If only adhesion of platelets to endothelium and not aggregation of platelets occurs, this can be followed by alteration of light transmission in the aggregometer. If platelets aggregate in addition to adhering to endothelium, adhesion can be monitored by phase-contrast microscopy. In each case, the platelets and endothelial cells will be examined by TEM and by FE if adhesion occurs. The above tests will be conducted both in the presence and absence of nicotine in order to determine whether this agent influences platelet-platelet or platelet-endothelial cell interaction. Further experiments will be conducted with PRP from patients congenitally deficient in fibrinogen, Factor VII, Factor VIII, Factor IX, Factor X, or Factor XII, with PRP from patients receiving dicumarol therapy, and with PRP from patients homozygous for von Willebrand's disease to determine any possible role of the

intrinsic coagulation system or von Willebrand's factor in the adhesion reaction. In the same manner, PRP from patients with thrombasthenia will be studied to determine reactivity of these defective platelets with endothelium. Similarly, studies will be conducted with platelets isolated by gel filtration and suspended in MTS in an attempt to identify cofactors for the reaction such as divalent cations or fibrinogen. The latter studies will be conducted by use of purified preparations of albumin, fibrinogen, and gamma G globulin. These studies will extend preliminary work reported in Appendix I.

c. Effects of enzymatic alteration of surface constituents of endothelial cells or platelets. Endothelial cells or isolated platelets in citrated MTS will be incubated at 37°C in the presence and absence of nicotine with low concentrations of the following: neuraminidase (1-100 units/ml), thrombin (0.01-10 Iowa units/ml), trypsin (.01-1 mg/ml), papain (0.1-10 mg/ml), plasmin (0.05-5 mg/ml), or collagenase (0.05-2.0 mg/ml). Following incubation the endothelial cells or platelets will be washed 4x in MTS, resuspended in MTS, and evaluated in the model system for the presence or absence of adhesion between cell types. Neuraminidase removes sialic acid thus reducing the overall net negative charge on cells. Trypsin and papain remove polypeptides and glycopeptides from the outer layers of the plasma membrane. Plasmin attacks fibrinogen, a platelet plasma membrane constituent which plays an important role in aggregation reactions. Collagenase is of interest primarily because of its use in recovery of endothelial cells from the umbilical cord vein. The enzyme preparations to be used will be purified but not entirely free of contaminants, a fact to be remembered in evaluation of data obtained in this work.

Polypeptides and glycopeptides liberated from both endothelial cells and platelets by treatment with enzymes, especially collagenase, thrombin, papain, and trypsin, will be characterized by polyacrylamide gel disc electrophoresis of supernates of enzyme-treated cells. Further, proteins obtained by SDS treatment of control and enzyme-treated cells can be characterized by electrophoresis in SDS containing gel. Proteins exposed on the outer surface of the endothelial cell or platelet plasma membrane can be labelled before exposure to enzymes to detect possible alteration in membrane geometry produced by enzyme treatment. This procedure will be used to determine whether the contractile protein of endothelial cells and platelets is exposed in part on the outer surfaces of these cells.

Such studies hopefully will form the basis for future work on identification of surface entities responsible for nonadhesion of endothelial cells and platelets and for the high degree of blood compatibility of the endothelial cell. Studies similar to those planned here have been reported previously for platelets[2] and for amoebae.[3] At the least we shall seek a correlation between loss of peptides and alteration of endothelial cell-platelet reactivity. At the most, we could gain a clear understanding of protein and saccharide components of the outer reaches of the platelet plasma membrane.

In addition, when sufficient numbers of endothelial cells are available, we shall isolate cell membrane fractions in the presence and absence of nicotine by a glycerol loading technique and characterize the component proteins before and after enzyme treatment by use of SDS polyacrylamide gel disc electrophoresis. Use of dithiothreitol with SDS will permit more refined critical evaluation of disc electrophoretic patterns and changes which may be produced in these. Staining gels with periodic acid-Schiff (PAS) will permit detection of alteration in glycoprotein components.

Enzyme-treated cells will be studied also by whole cell electrophoresis to detect alteration in surface charge and by TEM and FE to search for ultrastructural alterations. Especially sought for will be alterations of membrane associated particles seen with FE techniques. In later phases of the proposed work we plan to investigate effects of certain other enzymes on interaction of endothelial cells and platelets. Such enzymes include chymotrypsin, elastase, hyaluronidase, acid and alkaline phosphatase, and phosholipase C.

d. Effects of miscellaneous agents on endothelial cell-platelet interaction. In the model system, a number of diverse agents will be evaluated for their effect on the interaction of endothelial cells and platelets in the presence and absence of nicotine. These agents include: heparin, protamine, polylysine, Reptilase, Arvin, and early and late fibrin degradation products (FDP). Heparin has a strong negative charge in addition to being an effective anticoagulant; and protamine, being positively charged, is a potent heparin-neutralizing agent. Polylysine is a positively charged polymer which reacts with cell surfaces. Reptilase and Arvin alter fibrinogen. All of these agents including FDP affect blood coagulation, or platelets, or both, and therefore may well influence endothelial cell-platelet interaction. Our chief aim is to determine conditions under which the two cell types react.

e. Inhibition of endothelial cell-platelet adhesion. Under conditions in which endothelial cells and platelets are found to adhere, we plan to investigate the effects of a wide spectrum of agents which belong to the general class of antithrombotic drugs. Each agent will be incubated with endothelial cells or platelets at 37°C for 1 min and for 30 min prior to use of the treated cells in the model system. Agents to be tested include: aspirin, atropine, sulfinpyrazone, phenylbutazone, cyproheptadine, imipramine, reserpine, phentolamine, dibenzyline, cocaine, parachoromercuribenzoate, promethazine, and prostaglandin E_1. All of these agents, representing various pharmacologic classes, have been reported to inhibit platelet aggregation, and each will be tested in the concentration range 10^{-3} to 10^{-8} M. Effects of nicotine on agents inhibiting platelet-endothelial cell adhesion will be determined.

Agents which inhibit adhesion of endothelial cells and platelets will be evaluated further by means of TEM and FE for possible effects on ultrastructure of both cell types. Use of both TEM and FE has been found most helpful in past evaluation of antithrombotic agents.[4]

f. Further studies will include investigation of the possible role of leukocytes or erythrocytes in endothelial cell-platelet reactions in the model system. Little is known of possible interactions of leukocytes and platelets, although in the rabbit it has been shown that these cells can play a role in release of vasoactive amines from platelets.[5] Initial studies will involve addition of buffy coat components to the model system in the presence and absence of nicotine to determine whether leukocytes affect the platelet-endothelial cell reaction. Erythrocytes in small numbers will also be added to the model system for determination of their possible effects.

3. Investigation of Possible "Endothelial Supporting" Function of Platelets. Endothelial cells grown in tissue culture with or without addition of platelets to the culture medium will be evaluated for reactivity with isolated platelets or platelets in plasma in the model system. Studies will be conducted both in the presence and absence of nicotine. Protein patterns on gel disc electrophoresis will be compared using SDS-solubilized preparations of each of the two cell populations. Each cell population will be examined at intervals during growth for ultra-structural evidence of platelet incorporation into endothelial cells.

4. Studies of an Endothelial Cell Component Which Inhibits Platelet Aggregation. Endothelial cells recovered from umbilical cord vein contain a substance which inhibits platelet aggregation. This activity, possibly a polypeptide, is heat labile (90°C, 10 min) and has an apparent molecular weight in the range 1600 to 6000. This antiaggregating activity can be detected easily by use of the aggregometer.

a. Physical properties of the inhibitor. We propose to investigate further this activity by the following studies:

1) Preliminary purification studies by use of filtration through membranes of known porosity as well as use of classical ammonium sulfate fractionation and Sephadex G25 or G50 chromatography. We are limited somewhat by the small quantities of starting material available, but we can obtain even now samples of sufficient volume for preliminary work. Later it is hoped that sufficient quantities of tissue culture endothelial cells can be produced to permit further studies of the inhibitor. This inhibitory activity is released from endothelial cells by freeze-thawing or by treatment with mixtures of platelets and aggregating agents.

2) Gel disc electrophoresis of semipurified fractions to define contaminants and identify electrophoretic properties of the activity. Gel columns can be sliced to isolate component bands; each band or disc can be tested for antiaggregant activity by use of the aggregometer.

3) Further purification can be carried out by use of appropriate Sephadex chromatography and Geon-Pevicon electrophoresis. Further purification steps will depend on the nature of the inhibitory activity, its concentration in cells, and its stability.

b. Investigation of mechanisms of release of the inhibitor. Further studies will be carried out to define conditions under which the inhibitory activity is released from endothelial cells. The aggregating agents thrombin, ADP, epinephrine, serotonin, and collagen, in a wide concentration range (vida supra) will be incubated in the presence and absence of nicotine with freshly isolated endothelial cells and with endothelial cells grown in tissue culture. Subsequently, cells will be removed by centrifugation and the supernatant solutions assayed for ability to inhibit aggregation of platelets in plasma induced by standard concentrations of the aggregating agents. Cofactors for release of inhibitory acitivity such as divalent cations or certain plasma proteins will be sought. Homogenization of endothelial cells will be used to obtain 100% release of inhibitor. Inhibitors released by homogenization will be incubated with aggregating agents prior to their addition to platelets. Temporal studies will be conducted with a range of concentrations of aggregating agents and inhibitors to characterize the type of inhibition which occurs. Efforts will be made to determine effects of the inhibitor on aggregating agents and on platelets. Possible effects of low levels of nicotine on content and release of the inhibitor will be investigated as will effects of nicotine on the actions of the inhibitor. Endothelial cells will be examined by TEM following release of the inhibitor in an effort to determine intracellular storage sites. Platelets will be examined by TEM and FE before and after exposure to the inhibitor and a search will be made for ultrastructural alteration, especially in the plasma membrane.

c. Other possible effects of the antiaggregant activity. Antiaggregant activity released from endothelial cells will be tested for its effect on adhesion of platelets to glass or collagen, clot retraction, "phagocytosis," adhesion of platelets to endothelial cells, and the platelet release reaction. In addition possible effects of the inhibitor on the intrinsic blood coagulation system will be sought by mixture of the inhibitor with normal plasma. Tests will be conducted both in the presence and absence of nicotine in each case.

5. In Vivo Studies. In vivo studies of the interaction of endothelial cells and platelets and effects of nicotine on these interactions will be conducted in the microvasculature of the hamster cheek pouch. Later, if studies in a nonhuman primate are deemed necessary, similar studies can be conducted in the microcirculation of the mesentery of the spider monkey. The following investigations will be conducted:

a. Effects of nicotine, ADP, epinephrine, or serotonin applied to venules iontophoretically or of thrombin infused in dilute solution. Concentrations of aggregating agents will be chosen to produce a standard nonocclusive mural thrombotic deposit. Preliminary investigation indicates that this approach is feasible. Careful studies will be performed to determine optimal concentrations and rate of delivery for each aggregating agent. Effects of nicotine on formation of the standard mural thrombus will be studied. This model for mural thrombosis will be characterized thoroughly by TEM to elucidate relationships of endothelial cells, basement membrane and other vascular wall components, and platelets. The model will be based on endothelial cell-platelet adhesion, not connective tissue-platelet adhesion.

b. Effects of infused enzymes on the model for mural thrombosis. Infusion of low concentrations of neuraminidase, trypsin, papain, plasmin, Arvin, or collagenase will be made to determine if removal of sialic acid, surface glycopeptides, or fibrinogen will alter the mural thrombus model. Collagenase and trypsin will be tested primarily to determine effects they may have on reactivity of endothelial cell-platelet interaction and will be correlated with similar in vitro studies. If altered endothelial cell reactivity results from enzyme treatment, studies will be conducted to determine effects of nicotine on this altered reactivity.

c. Use of the mural thrombus model to test for effects of anticoagulant and antithrombotic agents. The antiaggregating activity of human endothelial cell origin will be assayed in the mural thrombus model in both hamster and spider monkey. If the inhibitory activity is effective under conditions of the test, the mural thrombus model could serve as a bioassay, and effects of nicotine on actions of the inhibitor in vivo would be sought. Additionally, heparin and the antithrombotic agents listed in "2.e." above will be tested for their effects on endothelial cell-platelet adhesion in the in vivo mural thrombus model. Anti-aggregants will be given systemically, and a wide concentration range of each agent will be investigated. Effects of test agents will be monitored by light microscopy, cinephotomicrography, and, in selected cases, by TEM.

B. Methods of Procedure.

We have entered into several aspects of research on endothelium during the past year. Dr. David Sharp, an intern and graduate student in experimental pathology in our department, spent the 1971-72 academic year working in the laboratory of Dr. G. V. R. Born in London. Under Dr. Born's supervision, Dr. Sharp has mastered the technique of iontophoresis[6] and learned to apply this in studies of the microcirculation of the hamster cheek pouch. Dr. Sharp has studied the ultrastructure of endothelium exposed to iontophoretically applied aggregating agents such as adenosine diphosphate (ADP).[7,8] In addition, he spent one month in the laboratory of Dr. Arfors in Uppsala learning additional techniques used in research on the microcirculation.[9]

Dr. Sabiha Saba, long a member of our research group, began in July, 1972 a study of endothelial cells recovered from several sources. She has successfully recovered endothelial cells from segments of canine aorta by use of low concentrations of trypsin. She has also recovered endothelial cells from human umbilical cord vein by use of collagenase and has used these cells in aggregometric studies with platelets in plasma and with isolated platelets. These studies, presented in an accompanying manuscript, show that platelets in plasma do not adhere to endothelial cells recovered from umbilical cord vein even if adenosine diphosphate (ADP) or epinephrine is added. Such endothelial cells do not adhere to platelets isolated from plasma even in the presence of added thrombin, ADP, or epinephrine. Further, human endothelial cells exposed to platelets and aggregating agents release a small molecule of apparent 1400 to 16000 molecular weight, possibly a polypeptide, which is an effective inhibitor of platelet aggregation. This inhibitor is inactivated at 90°C for 10 min.

We have established successful tissue culture of endothelium of human umbilical cord vein origin in our laboratory. This was accomplished by Dr. Saba and Dr. Mohammad. Dr. Ralph Nachman generously permitted Dr. Mohammad

to visit his laboratory and receive limited but highly useful instruction in tissue culture of endothelium. In addition, Drs. Saba and Mohammad received informal training in tissue culture techniques in the laboratory of Dr. D. O. Adams, Department of Pathology, Duke University, Durham, N. C.

Methods and materials are listed alphabetically:

1. Adhesion of platelets to collagen will be quantitated by addition of standard amounts of human collagen of Achilles tendon origin prepared by the method of Hovig[10] to PRP in the aggregometer. An increase in light transmission is produced by adhesion of platelets to collagen when ethylenediamine tetraacetic acid (1%) is used as anticoagulant. Results will be monitored by phase-contrast microscopy also.

2. Adhesion of platelets to glass will be quantitated by the method of George et al.[11]

3. Adenine nucleotide content of platelets will be measured by the luciferase reaction using a Biometer.[12]

4. Aggregating agents. Human thrombin prepared by the method of Miller and Copeland[13] will be further purified by the method of Kerwin and Milstone.[14] Human Achilles tendon will be used to prepare "collagen" by the method of Hovig. ADP, epinephrine, and serotonin will be purchased from Sigma Chemical Co. All aggregating agents will be dissolved in 0.08 M phosphate buffer, pH 7.35, in 0.145 M NaCl since Tris buffer produces undesirable ultrastructural changes.

5. Aggregometer. Three Chrono-Log aggregometers are in use in our laboratories.[15] Aggregometer tracings will be analyzed by the techniques of Frojmovic.[16] In addition, samples can be monitored by use of phase-contrast microscopy and, when warranted, by TEM.

6. Blood. Donors of normal blood will be carefully selected students and young employees of both sexes. Donors are rejected if they receive any type of medication regularly or if they have taken aspirin within the preceding week. Donors congenitally deficient in fibrinogen or blood coagulation factors VII, VIII, IX, X, or XII as well as patients with von Willebrand's disease or thrombasthenia are readily available through our Clinical Coagulation Center. Dogs congenitally deficient in factors VII, VIII, or IX also are readily available at the department's Frances Owen Blood Research Lab. Patients receiving dicumarol therapy are available through our Clinical Coagulation Laboratory.

7. Chromatography with Sephadex G25 or G50 will be conducted as described by Fisher.[17] Gel filtration chromatography of platelets will be carried out by the method of Tangen et al. using MTS as elution fluid.[18-20]

8. Clot retraction of PRP will be conducted by a previously described method.[4]

9. Electron microscopy. TEM of cells fixed in situ or in suspension will be carried out by previously described methods[21] using buffered 4/3 or 8/3% glutaraldehyde as fixative. FE methods have been described and are those of Reddick and Mason[22] and of Mühlethaler.[23] The Balzers freeze-etch apparatus[24] in the nearby Department of Botany is available to us on a rental basis.

10. Electrophoresis and Study of Proteins and Glycoproteins. Electrophoretic mobility of intact cells will be performed by the method of Seaman.[25] Disc electrophoresis in 5% or 7% polyacrylamide gels with barbital buffer and with or without addition of sodium dodecyl sulfate will be carried out as described by Gordon[26] using Comassie blue stain for protein and periodic acid-Schiff stain for glycoprotein. Molecular weight estimations will be carried out as described by Weber and Osborn[27] and by Mann et al.[28] Electrophoresis in Geon-Pevicon will be by the method of Muller-Eberhard.[29] Lactoperoxidase iodination of exposed membrane protein will be carried out by the method of Phillips and Morrison.[30]

11. Endothelial cells. Human endothelial cells will be recovered from umbilical cord vein by use of low concentrations of collagenase and from vena cava by use of dilute trypsin. Umbilical cords are being obtained daily from the Obstetrical Services of N. C. Memorial Hospital, Chapel Hill, N. C., Watts Hospital, Durham, N. C., and Alamance County Hospital, Burlington, N. C. Tissue culture of endothelial cells from cord or vena cava will be conducted by methods described by Nachman;[31] these have proven successful in our hands. Human vena cava segments can be obtained within one hour after death from cadavers authorized for organ donations.

12. Enzymes. Arvin (Twyford Labs, London) and plasmin (Schwarz-Mann) will be purchased from commercial sources. Collagenase, neuraminidase, papain, trypsin, and other listed enzymes will be purchased from Worthington Biochemicals.

13. Glassware will be coated with polyethylene oxide as described by Hiatt et al.[32] or with silicone as previously described.[33] Glow discharge treatment of glass will be carried out gratis at Research Triangle Institute, Research Triangle Park, N. C.

14. Iontophoresis will be carried out by methods described by Duling et al.[6]

15. Membrane preparations from endothelial cells will be obtained by the method of Barber and Jamieson.[34]

16. Miscellaneous. Antithrombotic agents are all on hand; additional supplies can be purchased from Sigma Chemical Co. or obtained gratis from appropriate pharmaceutical firms. Fibrin degradation products will be prepared by the method of Marder et al.[35] Heparin will be "Liquaemin" from Organon Labs. Reptilase (Pentapharm, Inc.) is on hand.

17. Microvasculature. Procedures used for study of the microvasculature of the hamster cheek pouch[36] or spider monkey mesentery[37] have been described. Animals will be housed in the medical school's Division of Laboratory Animal Medicine and all work with animals will be conducted in accordance with NIH regulations.

18. Nicotine as nicotine HCl will be prepared in 0.05 M phosphate buffer, pH 7.35 in 0.154 M NaCl.

19. Phagocytosis of latex particles by platelets will be conducted as described by Movat et al.[38]

20. Platelets will be prepared as suspensions in citrated plasma at 23°C as previously described.[33] Platelets separated from plasma by gel filtration will be prepared by the method of Tangen et al.[18] We have recently found these platelets superior to those prepared by a number of other methods.[39]

21. Release reaction of platelets will be followed by measurement of adenine nucleotides using the luciferase reaction,[12] or by use of C^{14} labelled serotonin.[40]

22. Tissue culture of endothelial cells will be conducted by methods described by Nachman.[31]

All of the above methods are being used or have been used by us except the iodination of cell membrane proteins.

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Our research group, currently occupying 2000 sq. ft. of research space will move in December, 1973 into 2800 sq. ft. of new research space in the Preclinical Education Building. This space will be equipped with fume hoods, walk-in temperature-controlled rooms, and an electron microscopy suite for our RCA-EMU3G transmission electron microscope and Quickscan scanning electron microscope. Our group now has on hand a variety of centrifuges including a Spinco preparative ultracentrifuge, various pH meters and balances, a Nuclear Chicago 3 channel scintillation counter, gel electrophoresis apparati of several types, equipment for column or thin layer chromatography, equipment for immunodiffusion, and immunoelectrophoresis, the Seaman apparatus for electrophoresis of intact cells, several phase-contrast microscopes, three aggregometers and recorders, gradient maker and ultraviolet monitors for chromatography, laminar flow hood and atmosphere-controlled incubator for tissue culture, an ultrasonic probe, drying ovens, various pumps and temperature-controlled baths, refrigerators, -20° and -40 to -70° freezers, vacuum evaporators, complete dark room facilities, micromanipulator and some necessary optics for micro-vascular studies, and a Zeiss PMQII spectrophotometer. Available within the department are complete facilities for amino acid analysis (Beckman) and for column electrophoresis and electrofocusing (LKB).

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s) even if no salary requested)	% time	Amount (including social security, retirement, and insurance)
Mason, R. G., M.D., Ph.D., Principal Investigator	10	---
Saba, S. R., M.D., Coinvestigator	30	3846
Chuang, H. Y. K., Ph.D., Coinvestigator	20	3712
Mohammad, S. F., Ph.D., Coinvestigator	30	3502
Sharp, D. E., M.D., Coinvestigator	20	---
Reddick, R. L., M.D., Coinvestigator	20	---
Technical		
Perry, J. M. (technician)	100	9652
Bazan, M. E. (lab helper)	50	3050
Smith, M. (secretary)	25	1770

Sub-Total for A 25,532

B. Consumable supplies (by major categories)

General chemicals	700
Biochemicals including enzymes	1600
Human blood (50 units at $15/unit)	750
Tissue culture supplies	1800
Electron microscopy and photographic supplies	800
Animal purchase and board	480

Sub-Total for B 6130

C. Other expenses (itemize)

Mileage ($0.10/mile; 2000 miles/yr for procurement of umbilical cords from cooperating hospitals)	200
Copying	100
Communications	100
Glass shop and electronics shop charges	200
Service contracts (scintillation counter, 25%, $225; electron microscope, 25%, $900)	1125

Sub-Total for C 1725

Running Total of A + B + C 33,387

D. Permanent equipment (itemize)

Payton aggregometer	1900
Beckman recorder	1100
Miscellaneous (trip balance, table top centrifuge, water bath)	600

Sub-Total for D 3600

E. Indirect costs (15% of A+B+C) 15% of $33,387 E 5008

Total request 41,995

15. Estimated future requirements:

	Salaries (+10% yearly)	Consumable Suppl. (+5% yearly)	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	28,085	6,437	1725	1000	5437	42,684
Year 3	30,894	6,759	1725	1000	5907	46,285

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

	Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
1.	Role of plasmatic components in blood-solid surface reactions	NHLI, NIH 5K4HE46351	$25,000/yr	July, 1970 to July 197
2.	Blood platelets, membranes, aggregation and adhesion	NHLI, NIH, HL13296	$21,031	June 1971 to June 197€
3.	Mechanisms of platelet adhesion and aggregation in thrombosis	NHLI, NIH, HL14228	$73,433	June 1971 to Dec. 197€
4.	Antithrombogenic surfaces: platelet-interface reactions	AKCUP, NIAMDD, NIH PH4368977 (contract)	$87,358	Dec. 1972 to Dec. 197

PENDING OR PLANNED

	Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
1.	Pathologic alterations in endothelium and thrombosis	NHLI, NIH	51,866 (requested)	Jan. 1974 to Jan. 197 (requested)

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to
University of North Carolina

Mailing address for checks
W. W. Falk
Contract Administration
South Bldg., Chapel Hill

Principal investigator
Typed Name R. G. Mason
Signature R. G. Mason Date August 4/15/73
Telephone 919 966 1348
Area Code Number Extension

Responsible official of institution A H Shepard, Jr. 23 Aug 1973
Typed Name Mr. A. H. Shepard, Jr., Asst. Vice President and Treasurer
G. R. Holcomb, Ph.D., Dean, Res. Admin.
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Area Code Number Extension

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